

**MICROBIAL POLYNUCLEOTIDES EXPRESSED  
DURING INFECTION OF A HOST**

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**GOVERNMENT INTERESTS**

This invention was made with Government support under Grant Number DE11857 awarded by the National Institute of Dental and Craniofacial Research. The Government has certain rights in the invention.

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**PRIORITY**

This application is a Section 371 National Stage Application of International Application No. PCT/US00/2134, filed August 4, 2000, which claims priority from provisional application 60/147,551, filed August 6, 1999, which is herein incorporated by reference.

**TECHNICAL AREA OF THE INVENTION**

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This invention provides methods and compositions for identifying and obtaining microbial polynucleotides expressed during infection of a host and the use of these polynucleotides in the diagnosis, treatment, and prevention of microbial infections.

**BACKGROUND OF THE INVENTION**

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Microbial infections are complex, dynamic processes that evolve constantly within the host. In many instances, virulence gene expression is modulated in response to the changing environment encountered at the site of infection. Mekalanos, *J. Bacteriol.* 174:1-7 (1992); Mahan *et al. Science* 259:686-688 (1993). It is unlikely that all regulated virulence determinants of a pathogen can be identified *in vitro* because it is technically impossible to determine and

mimic all of the complex and changing environmental stimuli that occur at the site of an infection. Pioneering technologies such as *in vivo* expression technology (IVET), signature-tagged mutagenesis (STM) and differential fluorescence induction (DFI) were designed to identify genes specifically expressed *in vivo*, and fill the gaps in our understanding of microbial pathogenesis. Handfield & Levesque, *FEMS Microbiol. Rev.* 23:69-91 (1999); Heithoff *et al.*, *Trends Microbiol.* 5:509-513 (1997); Chiang *et al.*, *Ann. Rev. Microbiol.* 53:129-154 (1999); Hautefort & Hinton, *Phil. Trans. R. Soc. London Ser. B*, 255:601-611 (2000). All of these methods depend on a reasonable assumption: namely, that genes which are specifically induced during *in vivo* growth are likely to be important to the pathogenic process.

Although remarkably powerful, all of these technologies have certain limitations. Their major drawback is that they depend on the use of animal models of infection; animal models are not available for many pathogens and, in those cases where an animal model is available, it might not closely approximate the human condition. Consequently, a number of examples exist in the literature of erroneous conclusions being drawn by extrapolation of results from animal models to human infections. Smith, *Trends Microbiol.* 6:239-243 (1998). Further, many of these schemes are not readily applicable to genetically “undomesticated” microbes, that is microbes for which there is no well established or reliable means for genetic manipulation. Additionally, IVET, STM, and related technologies are technically difficult, and therefore restrict analysis to a single representative strain of the microbe and to only one or several time points in the infection process.

An example of a microbe that is not easily characterized by IVET, STM, and related technologies is *Actinobacillus actinomycetemcomitans* (*Aa*), an etiologic agent of periodontal diseases. *Aa* has emerged as one of the best examples of a bacterium demonstrating a direct

correlation with disease manifestation in the oral cavity. *Aa* is known to be the etiologic agent for localized juvenile periodontitis (LJP). See, Slots & Ting, *Periodontol.* 2000, 20:82-121 (1999). Despite the fact that some *Aa* virulence factors have been identified, other important contributors to the infectious process have yet to be identified.

## 5 SUMMARY OF THE INVENTION

It is an object of the invention to provide methods for identifying polynucleotides useful for vaccine design, diagnostics, and antibiotherapy. It is a further object of the invention to provide methods and compositions useful in vaccine design, diagnosis, and therapy of *A. actinomycetemcomitans* infection. This and other objects of the invention are provided by one or  
10 more of the embodiments described below.

The invention provides methods useful for identifying polynucleotides expressed by a microbe during infection of a host. One embodiment provides a method of identifying a polynucleotide of a microbe that is expressed *in vivo*. Antibodies against antigens that are expressed by the microbe *in vivo* and *in vitro* are adsorbed with cells or cellular extracts of the  
15 microbe that have been grown *in vitro*. Unadsorbed antibodies are isolated and are probed against an expression library of the microbe's DNA or RNA. A polynucleotide of the microbe that is expressed *in vivo* is isolated and identified.

Another embodiment of the invention provides a method of comparing polynucleotides of a microbe that are expressed *in vivo* at different stages of infection of the microbe. A first sera  
20 sample from one or more hosts infected with or previously infected with the microbe is adsorbed with cells or cellular extracts of the microbe that have been grown *in vitro*. Each host is in about the same stage of the infection. A second sera sample from one or more hosts infected with or previously infected with the microbe is adsorbed with cells or cellular extracts of the microbe

that have been grown *in vitro*. Each host is in about the same stage of the infection and the stage of the infection is different from the stage of infection in the first sample. Unadsorbed antibodies from the first serum sample and from the second serum sample are isolated. A first expression library of the microbe is probed with the unadsorbed antibodies from the first serum sample, and  
5 a second expression library is probed with the unadsorbed antibodies from the second serum sample. The first and second expression libraries can be the same expression library. Polynucleotides of the microbe that are expressed *in vivo* are identified for the first and second serum sample. The polynucleotides of the microbe that are expressed *in vivo* at different stages of infection of the microbe are compared.

- 10 Still another embodiment of the invention provides a method of comparing polynucleotides of a microbe that are expressed *in vivo*, wherein the microbe has infected its host by different routes of infection. A first sera sample from one or more hosts infected with or previously infected with the microbe is adsorbed with cells or cellular extracts of the microbe that have been grown *in vitro*. Each host has been infected by about the same route of infection.
- 15 A second sera sample from one or more hosts infected with or previously infected with the microbe are adsorbed with cells or cellular extracts of the microbe that have been grown *in vitro*. Each host has been infected by about the same route of infection, and the route of infection is different from the route of infection for the first sample. Unadsorbed antibodies are isolated from the first serum sample and from the second serum sample. A first expression library of the  
20 microbe is probed with the unadsorbed antibodies from the first serum sample, and a second expression library is probed with the unadsorbed antibodies from the second serum sample. The first and second expression libraries can be the same expression library. The polynucleotides of the microbe that are expressed *in vivo* are identified for the first and second serum sample and

the polynucleotides of the microbe that are expressed *in vivo* by different routes of infection of the microbe are compared.

Another embodiment of the invention provides a method of confirming an animal model of microbial infection as a valid model. A first sera sample from one or more animal model hosts  
5 infected with or previously infected with a microbe, is adsorbed with cells or cellular extracts of the microbe that have been grown *in vitro*. A second sera sample from one or more second hosts infected with or previously infected with the microbe, is adsorbed with cells or cellular extracts of the microbe that have been grown *in vitro*, where the second host is a different species of animal than the animal model host. Unadsorbed antibodies are isolated from the first serum  
10 sample and from the second serum sample. A first expression library of the microbe is probed with the unadsorbed antibodies from the first serum sample, and a second expression library is probed with the unadsorbed antibodies from the second serum sample. The first and second expression libraries can be the same expression libraries. Polynucleotides of the microbe that are expressed *in vivo* are identified for the first and second serum sample and polynucleotides of the  
15 microbe that are expressed *in vivo* in the animal model host and the second host are compared. If the polynucleotides expressed *in vivo* in the animal model and in the second host are the same or similar, then the animal model is confirmed as a valid model.

Yet another embodiment of the invention provides a purified polypeptide comprising at least 5 contiguous amino acids of an amino acid sequence selected from the group consisting of  
20 SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20.

Even another embodiment of the invention provides a purified polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20.

Another embodiment of the invention provides a purified polynucleotide comprising at least about 15 contiguous nucleic acids of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

Yet another embodiment of the invention provides a purified polynucleotide comprising a sequence selected from the group consisting SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

Another embodiment of the invention provides a method for identifying the presence of a first polynucleotide. A sample suspected of containing the first polynucleotide is contacted with a second polynucleotide comprising at least about 15 nucleotides of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19, under hybridization conditions. The presence of a hybridized first and second polynucleotide complex is detected and the presence of the first polynucleotide is identified.

Still another embodiment of the invention provides a method of eliciting an immune response. A purified polypeptide comprising at least about 5 contiguous amino acids of an amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:12, SEQ

ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20 is administered to an animal.

An immune response is elicited.

Even another embodiment of the invention provides a method of treating, preventing, or ameliorating a disease or infection caused by *A. actinomycetemcomitans*. A purified polypeptide comprising at least about 5 contiguous amino acids of an amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20 is administered to an animal. The disease or infection is treated, prevented, or ameliorated.

The invention thus provides methods for identifying antigens expressed during an actual microbial infection that do not rely on animal models. The methods can be used with microbes for which there is no well established or reliable means for genetic manipulation and allow for the analysis of a pool of strains of a microbe that represent all of the various clinically important clonotypes. The methods of the invention further provide a technique of defining a timeline for antigen production by the microbe throughout the course of a natural infection process. The staging of individual *in vivo* induced antigens provides relevant information on the pathogenesis of the microorganism and on the host response to the infection. This in turn provides valuable insights for rationale vaccine design, diagnostics, and antibiotherapy.

The combination of screening, confirmation, and staging methods of the invention to identify and test *in vivo* induced antigens is far superior to existing *in vivo* expression technologies by virtue of its efficiency and direct assessment in natural human infections. The methods are rapid and simple, which renders the screening of a whole genome possible in a timely fashion.

The invention further provides for the first time methods for a comprehensive analysis of the expression of infection-related antigens at the genomic level of different clinical strains of a microbe and of different stages of infection in the host.

5 Additionally, polynucleotides isolated by the methods of the invention can advantageously be studied by conventional techniques, including mutant analysis in animal models, with the confidence that the polynucleotide is actually expressed in infections, which is not the case with other available technologies.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows an *in vivo* induced antigen technology (IVIAT) scheme. IVIAT can begin  
10 with serum from one or more patients who have or recently had the disease or infection of interest. Such sera are individually tested to verify that they exhibit measurable antibody titers versus the microbial pathogen. One or more such reactive serum samples are adsorbed with cultured cells and lysates of the pathogen to remove antibodies reactive with antigens made during *in vitro* growth. The resulting adsorbed serum still contains antibodies reactive with  
15 immunogenic proteins produced by the pathogen only during *in vivo* growth. It is used to probe an expression library of the pathogen's DNA or RNA cloned in a convenient host. Reactive clones are isolated, and the cloned insert is sequenced. If more than one open reading frame is present on an insert, each is individually subcloned and retested with the adsorbed serum to identify which one encodes the reactive antigen. For independent verification, the  
20 polynucleotide is overexpressed, and the resulting recombinant protein is purified and used to raise monospecific antibodies. The antibodies are used to probe biological samples taken from patients infected with the pathogen. Reactivity with cells of the pathogen in clinical samples, but



not with cells of the pathogen grown *in vitro*, provides direct confirmation that the antigen is expressed only during *in vivo* growth.

Figure 2 shows sequential adsorption of pooled serum to eliminate antibodies reactive with *in vitro* expressed antigens. The pooled sera were subjected to five successive adsorptions with whole cells, two adsorptions with French press extracts, and two adsorptions with heat denatured French press extracts.

Figure 3 shows the probing of a partial genomic library. Plates containing appropriate numbers of colonies were replicated onto medium with IPTG to induce expression of the cloned fragments. The colonies were lifted onto duplicate membranes, which were then reacted with either the pooled unadsorbed (panel A) or pooled adsorbed (panel B) sera. Reactive clones were visualized by probing with peroxidase-conjugated goat anti-human immunoglobulin as a secondary reagent followed by development with a chemiluminescent substrate and autoradiography.

Figure 4 shows specific *in vivo* induction of Aa2008. Plaque samples were thawed on ice, homogenized by brief vortexing at 4°C, and immediately heat-fixed on microscope slides. Plaque samples and matched *in vitro* grown *A. actinomycetemcomitans* isolates taken from selective medium (Slots, *Clin. Microbiol.* 15:606 (1982)) were probed in parallel with FITC-labeled monospecific antibody directed against antigen Aa2008 and the TR-labeled *A. actinomycetemcomitans*-specific monoclonal antibody. Samples were examined by fluorescence microscopy (excitation wavelength 488 nm). Double color analysis, using matched differential emission filters, was performed to identify individual cells that were simultaneously labeled with FITC and TR. Bacterial cells in plaque that reacted with TR-labeled anti-*A. actinomycetemcomitans* monoclonal antibody (panel A) also reacted with FITC-labeled anti-

Aa2008 antibody (panel B) and these signals were found to colocalize (panel C). In contrast, matched *in vitro* grown *A. actinomycetemcomitans* reacted with the TR-labeled anti-*A. actinomycetemcomitans* monoclonal antibody (panel D) but not with FITC-labeled anti-Aa2008 antibody (panel E) and therefore no colocalization was observed (panel F).

5           Figure 5 shows western blot analysis of immunoreactivity of patient and control subjects' sera to Aa2008. Western blots of the inclusion body preparation of Aa2008 were probed using individual, unadsorbed sera from 20 periodontitis patients and 9 control subjects.

Figure 6 shows Table 2, which demonstrates confirmation and characterization of the IVIAT subclones.

10           Figure 7 shows Table 1, which provides a characterization of the IVIAT open reading frames. "\*" is a putative ribosome binding site.

## SEQUENCE LISTING

This application contains a sequence listing submitted on compact disk. The sequence listing submitted on compact disc, in compliance with 37 C.F.R. § 1.52(e)(5), is incorporated  
15           herein by reference. Two separate compact discs have been submitted, each containing the file "00-505 ST25" (26,624 bytes in size), and each created on March 16, 2006.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for identifying and isolating polynucleotides that are expressed only during *in vivo* growth of a microbe or pathogen. Briefly,  
20           the methods, termed *in vivo* induced antigen technologies (IVIAT), comprise obtaining a sample of antibodies against antigens that are expressed by the microbe *in vivo* and *in vitro* and adsorbing these antibodies with cells or cellular extracts of the microbe that have been grown *in vitro*. An example of a sample of antibodies that can be used in the invention is sera from

patients who have been or are infected with the microbe. *See, e.g.* Figure 1. The unadsorbed antibodies are isolated and are used to probe an expression library of the microbe's DNA or RNA. Reactive clones can be isolated and the cloned fragments sequenced.

### **IVIAT Methodology**

5           A sample of antibodies against antigens that are expressed by a microbe *in vivo* and *in vitro* is collected. The sample can comprise the serum of a host or hosts infected with or previously infected with the microbe. Because a host from which serum is collected has undergone or is actively engaged in an actual infection by the microbe of interest, the host's serum contains antibodies produced against microbial antigens expressed during the *in vivo*  
10   infectious process. Serum from an individual host may be used or pooled sera from two or more hosts can be used. For example, sera from about 2, 5, 25, 100, 500, or 1,000 hosts may be pooled for use in the instant invention.

          The optimum number of serum samples to use depends on the type of information that is being sought. If the main interest is in targets for vaccines and diagnostics, one or several sera  
15   samples should suffice to identify those proteins that are immunogenic in most individuals. On the other hand, if the main interest is a more detailed analysis of the potential molecular mechanisms used by the pathogen, the use of a larger pool of sera would reduce the possibility of missing genes or polynucleotides of interest due to variability in the human immune response. In cases where the pathogen causes protracted disease, pooled serum from patients in early, middle  
20   and late stages of infection or sequential samples taken from patients during acute and convalescent phases of infection would offer the best chance of identifying transiently expressed *in vivo* induced proteins. Also, certain pathogens can infect humans by more than one route (*e.g.*, via a wound, gastrointestinal tract, respiratory tract, or skin); in such cases, selective

pooling of serum from patients infected by various routes may enable the identification of route-specific *in vivo* induced proteins. In cases where different clonal variants or strains of the microbe cause disease, selective pooling of serum from patients infected by various clonal variants or strains can enable the study of the pathogenesis of each variant or strain.

- 5           A host may be any kind of animal. For example, hosts can comprise humans, baboons, chimpanzees, macaques, cattle, sheep, pigs, horses, goats, dogs, cats, rabbits, guinea pigs, rats, mice, chickens, ducks, fish, and shellfish.

- The microbe or pathogen can be any kind of a bacterium, a virus, a parasite, a prion, or a fungus. For example, the microbe can be *Candida*, *Aspergillus*, *Sporothrix*, *Blastomyces*,  
10   *Histoplasma*, *Cryptococcus*, *Pneumocystis*, *Coccidioides*, *Tinea*, *Toxoplasma*, *Plasmodium*, *Pseudomonas*, *Actinobacillus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Listeria*, *Corynebacterium*, *Actinomyces*, *Mycoplasma*, *Nocardia*, *Bordetella*, *Brucella*, *Francisella*, *Legionella*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, *Shigella*, *Streptococcus*, *Yersinia*, *Vibrio*, *Campylobacter*, *Helicobacter*, *Bacteroides*, *Chlamydia*, *Borrelia*, *Treponema*,  
15   *Leptospira*, *Aeromonas*, *Rickettsia*, *Ascaris*, *Cryptosporidium*, *Cyclospora*, *Entamoeba*, *Giardia*, *Shistosoma*, *Trypanosoma*, herpes virus, cytomegalovirus, Epstein-Barr virus, hepatitis virus, adenovirus, papillomavirus, polyomavirus, enterovirus, rotavirus, influenza virus, paramyxovirus, rubeola virus, rhabdovirus, human immunodeficiency virus, arenavirus, rhinovirus, and reovirus. The microbe or pathogen can also be any kind of veterinary microbe or  
20   pathogen. See e.g., Veterinary Microbiology, Hirsh & Zee, Eds., Blackwell Science, Inc., Malden, MA, 1999; Clinical Veterinary Microbiology, Quinn, Ed., Wolfe Publishing, London, (1994).

Antibodies which bind to antigens that are produced during *in vitro* propagation of the microbe of interest are eliminated from the sample of antibodies against antigens that are expressed by the microbe *in vivo* and *in vitro*. The antibodies that are produced *in vitro* can be removed from the sample by, for example, adsorption. Preferably, a sample containing

5 antibodies against antigens that are expressed by the microbe *in vivo* and *in vitro*, such as a serum sample of an infected host, are contacted with *in vitro* grown whole cells, cell extracts or both of the microbe, or whole cells, extracts of whole cells, or both of cells that are infected with the microbe of interest, *e.g.* a prokaryotic or eukaryotic cell infected with a virus or parasite.

All or substantially all of the antibodies in the antibody sample whose corresponding

10 antigens are derived from *in vitro* grown microbes will bind to these antigens to form immune complexes. However, antibodies directed against antigens that are specifically expressed during the *in vivo* infectious process will remain uncomplexed since their corresponding antigens are not present in the *in vitro* grown cells and/or cell extracts. The adsorption step can be performed by, for example, contacting the antibody sample with whole cells and/or cell extracts that are

15 immobilized on a solid support, such as a nitrocellulose membrane. *See, Brady & Daphtary, J. Infect. Dis.* 158:965-972 (1988). Optionally, the whole cell and/or cell extract sample can be denatured before use to expose additional immunoreactive epitopes. Several successive adsorptions can be performed using the same or different adsorption methodologies.

After the adsorption step or steps, unbound antibodies are separated from the antibody-

20 antigen complexes. After elimination of antibodies produced in response to microbial antigens expressed *in vitro*, the sample will comprise antibodies produced in response to antigens expressed *in vivo*. This sample can then be used to screen one or more, same or different, genomic expression libraries, for example, plasmid or bacteriophage genomic expression

libraries, of the microbe of interest. Methods of constructing genomic expression libraries are well known in the art. *See, e.g.,* Ausubel (ed.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. (1994); T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y. (1989).

- 5    Optionally, DNA or RNA from more than one strain or clonal variant of the microbe of interest can be pooled for expression library construction. Methods for screening these libraries with one or more labeled or unlabeled antibody-containing samples and for identifying and isolating clones from the library that encode the reactive antigens of interest are also well known in the art, *e.g.,* colony blotting methods *See, e.g.,* Ausubel (1994); Maniatis (1989).
- 10    Reactive clones identified by screening expression libraries of the microbe of interest can be characterized by conventional analysis. For example, the clone can be sequenced and the presence of open reading frames can be predicted, for example, using Fickett's, start/stop codon or other methods. *See, e.g.,* Fickett, *Nucleic Acids Res.* 10:5303-5318 (1982); Solovyev, *Nucleic Acids Res.* 22:5156-5163 (1994); Saqi, *Protein Eng.* 8:1069-1073 (1995); Ladunga and Smith,
- 15    *Protein Eng.* 20:101-110 (1997); Birney, *Nucleic Acids Res.* 24:2730-2739 (1996).

- Identification of signal sequences, particularly likely ribosome binding sites and transcription termination sequences, can also be made since the information provided by these analysis can be helpful in prioritizing the subcloning of open reading frames if more than one is present on the cloned insert. Moreover, the obtained sequence information can be used to
- 20    identify sequence similarities to known polynucleotides, *e.g.,* by BLAST analysis, and possible relationships to proven or putative virulence factors can be evaluated. In instances where more than one open reading frame is present on a cloned insert, all of them may be analyzed for their

ability to express *in vivo* induced antigens. For example, each open reading frame can be independently subcloned and tested with the adsorbed serum to identify *in vivo* induced antigens.

*In vivo* induced antigens identified by IVIAT can be directly verified as actually expressed by the microbe at the site of infection in the host by directly probing biological samples taken from disease sites of infected patients by any method known in the art. For example, a polypeptide which is expressed from a clone suspected of expressing an *in vivo* induced antigen can be used to raise polyclonal antibodies in mice. Optionally, the polynucleotide encoding the suspected antigen can be overexpressed and the resulting polypeptide purified and used to raise polyclonal antibodies. The antibodies can be labeled with, for example, fluorescein isothiocyanate (FITC). A biological sample from a disease site of a host infected with the microbe of interest is obtained. The biological sample and a matched *in vitro* grown sample of the microbe are assayed by, for example, immunofluorescence microscopy. The labeled antibodies will react with the microbe found in the biological sample, but will not react with *in vitro* grown cells. These results can provide direct evidence that the microbe expresses the IVIAT antigen exclusively during *in vivo* growth.

Once an antigen has been identified and confirmed as an *in vivo* induced antigen, the purified antigen can be used to screen the sera of individual patients for reactive antibodies. For example, a serum sample can be obtained from a patient suspected of having a particular microbial infection. The sample can be probed for reactive antibodies with a labeled, purified antigen.

For those microbes that actually have a validated animal model, the polynucleotides identified by IVIAT can also be studied by conventional techniques, including mutant analysis, with the *a priori* confidence that the polynucleotide is actually expressed and relevant in host

infections. IVIAT can also be used to validate *in vitro* and animal models by identifying those that reproduce the same or similar expression pattern seen with IVIAT in humans or other animal hosts. These confirmed models can then be used for further conventional analysis of the pathogen.

## 5 **Polypeptides**

Polypeptides of the invention can either be full-length polypeptides or fragments of polypeptides. For example, fragments of polypeptides of the invention can comprise about 5, 10, 25, 50, 100, 200, 250, 500, 750, or 1,000 amino acids of polypeptides of the invention. Examples of polypeptides of the invention include *Actinobacillus Actinomycetemcomitans* (*Aa*)

10 SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. Homologous amino acid sequences which are at least about 75, preferably about 90, 96, 98, or 99% identical to the nucleotide sequences shown in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20 are also *Aa* polypeptides.

Percent sequence identity has an art recognized meaning and there are a number of

15 methods to measure identity between two polypeptide or polynucleotide sequences. See, e.g., Lesk, Ed., *Computational Molecular Biology*, Oxford University Press, New York, (1988); Smith, Ed., *Biocomputing: Informatics And Genome Projects*, Academic Press, New York, (1993); Griffin & Griffin, Eds., *Computer Analysis Of Sequence Data, Part I*, Humana Press, New Jersey, (1994); von Heinje, *Sequence Analysis In Molecular Biology*, Academic Press,

20 (1987); and Gribskov & Devereux, Eds., *Sequence Analysis Primer*, M Stockton Press, New York, (1991). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux *et al.*, *Nuc. Acids Res.* 12:387 (1984)), BLASTP, BLASTN, FASTA (Atschul *et al.*, *J. Molec. Biol.* 215:403 (1990)), and



Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) which uses the local homology algorithm of Smith and Waterman (*Adv. App. Math.*, 2:482-489 (1981)). For example, the computer program ALIGN which employs the FASTA algorithm can be used, with an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2.

When using any of the sequence alignment programs to determine whether a particular sequence is, for instance, about 95% identical to a reference sequence, the parameters are set such that the percentage of identity is calculated over the full length of the reference polynucleotide and that gaps in identity of up to 5% of the total number of nucleotides in the reference polynucleotide are allowed.

Polypeptides of the invention further comprise biologically functional equivalents of at least about 5, 10, 25, 50, 100, 200, 250, or 500 amino acids of the polypeptides shown in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. A polypeptide is a biological equivalent if it reacts about the same to a polypeptide of the invention in an assay such as an immunohistochemical assay, an ELISA, an RIA, or a western blot assay. Preferably, the assay is a competition assay wherein the biologically equivalent polypeptide is capable of reducing binding of the polypeptide of the to a corresponding reactive antigen or antibody by about 80, 95, 99, or 100%.

Polypeptides of the invention preferably comprise an antigen that is recognized by an antibody reactive against the microbe of interest. The antigen preferably comprises one or more epitopes (or antigenic determinants). An epitope can be a linear, sequential epitope or a conformational epitope. Epitopes within a polypeptide of the invention can be identified by several methods. See, e.g., U.S. Patent No. 4,554,101; Jameson & Wolf, *CABIOS* 4:181-186

(1988). For example, a polypeptide of the invention can be isolated and screened. A series of short peptides, which together span the entire polypeptide sequence, can be prepared by proteolytic cleavage. By starting with, for example, 100-mer polypeptide fragments, each fragment can be tested for the presence of epitopes recognized in an enzyme-linked immunosorbent assay (ELISA). For example, in an ELISA assay a microbial polypeptide, such as a 100-mer polypeptide fragment, is attached to a solid support, such as the wells of a plastic multi-well plate. A population of antibodies are labeled, added to the solid support and allowed to bind to the unlabeled antigen, under conditions where non-specific adsorption is blocked, and any unbound antibody and other proteins are washed away. Antibody binding is detected by, for example, a reaction that converts a colorless substrate into a colored reaction product. Progressively smaller and overlapping fragments can then be tested from an identified 100-mer to map the epitope of interest.

Preferably, a polypeptide of the invention is produced recombinantly. A polynucleotide encoding a polypeptide of the invention can be introduced into a recombinant expression vector, which can be expressed in a suitable expression host cell system using techniques well known in the art. A variety of bacterial, yeast, plant, mammalian, and insect expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding a polypeptide can be translated in a cell-free translation system.

If desired, a polypeptide can be produced as a fusion protein, which can also contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase, histidine tag, and staphylococcal protein A. More than one polypeptide of the invention can be present in a fusion protein.

### **Polynucleotides**

Polynucleotides of the invention contain less than an entire microbial genome and can be RNA or single- or double-stranded DNA. Preferably, the polynucleotides are purified free of other components, such as proteins and lipids. The polynucleotides of the invention encode the polypeptides described above. Polynucleotides of the invention can also comprise other nucleotide sequences, such as sequences coding for linkers, signal sequences, heterologous signal sequences, TMR stop transfer sequences, transmembrane domains, or ligands useful in protein purification such as glutathione-S-transferase, histidine tag, and staphylococcal protein A.

Degenerate nucleotide sequences encoding the polypeptides of the invention, as well as homologous nucleotide sequences which are at least about 75, preferably about 90, 96, 98, or 99% identical to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19 and the complements thereof are also polynucleotides of the invention. Percent sequence identity can be calculated as described in the "Polypeptides" section. Complementary DNA (cDNA) molecules, species homologs, and variants of *Aa* polynucleotides which encode biologically active *Aa* polypeptides also are *Aa* polynucleotides. Preferably, a polynucleotide of the invention comprises about 5, 10, 15, 50, 100, 500, or 1,000 nucleotides of a nucleic acid sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

Polynucleotides of the invention can be isolated from nucleic acid sequences present in, for example, a biological sample, such as plaque, saliva, sputum, blood, urine, feces,

cerebrospinal fluid, amniotic fluid, wound exudate, or tissue, from an infected individual.

Polynucleotides can also be synthesized in the laboratory, for example, using an automatic synthesizer. An amplification method such as PCR can be used to amplify polynucleotides from either genomic DNA or cDNA encoding the polypeptides.

- 5 Polynucleotides of the invention can comprise coding sequences for naturally occurring polypeptides or can encode altered sequences which do not occur in nature. If desired, polynucleotides can be cloned into an expression vector comprising, for example, promoters, enhancers, or other regulatory elements that drive expression of the polynucleotides of the invention in host cells. An expression vector can be, for example, a plasmid, such as pBR322,  
10 pUC, or ColEI, or an adenovirus vector, such as an adenovirus Type 2 vector or Type 5 vector. Optionally, other vectors can be used, including but not limited to Sindbis virus, simian virus 40, alphavirus vectors, poxvirus vectors, and cytomegalovirus and retroviral vectors, such as murine sarcoma virus, mouse mammary tumor virus, Moloney murine leukemia virus, and Rous sarcoma virus. Minichromosomes such as MC and MC1, bacteriophages, phagemids, yeast artificial  
15 chromosomes, bacterial artificial chromosomes, virus particles, virus-like particles, cosmids (plasmids into which phage lambda *cos* sites have been inserted) and replicons (genetic elements that are capable of replication under their own control in a cell) can also be used.

- A vector comprising a polynucleotide of the invention can be transformed into, for example, bacterial, yeast, insect, or mammalian cells so that the polypeptides of the invention  
20 can be expressed in and isolated from cell culture. Any of those techniques which are available in the art can be used to introduce polynucleotides into the host cells. These include, but are not limited to, transfection with naked or encapsulated nucleic acids, cellular fusion, protoplast fusion, viral infection, and electroporation.

Polynucleotides of the invention can be used, for example, as probes or primers to detect the presence of microbial polynucleotides in a sample, such as a biological sample. The ability of such probes to specifically hybridize to microbial polynucleotide sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample.

- 5 Polynucleotide probes of the invention can hybridize to complementary sequences in a sample such as a biological sample, including plaque, saliva, sputum, blood, urine, feces, cerebrospinal fluid, amniotic fluid, wound exudate, or tissue. Polynucleotides from the sample can be, for example, subjected to gel electrophoresis or other size separation techniques or can be dot blotted without size separation. The polynucleotide probes are preferably labeled. Suitable  
10 labels, and methods for labeling probes are known in the art, and include, for example, radioactive labels incorporated by nick translation or by kinase, biotin, fluorescent probes, and chemiluminescent probes. The polynucleotides from the sample are then treated with the probe under hybridization conditions of suitable stringencies.

- Depending on the application, varying conditions of hybridization can be used to achieve  
15 varying degrees of selectivity of the probe towards the target sequence. For applications requiring high selectivity, relatively stringent conditions can be used, such as low salt and/or high temperature conditions, such as provided by a salt concentration of from about 0.02 M to about 0.15 M salt at temperatures of from about 50°C to about 70°C. For applications requiring less selectivity, less stringent hybridization conditions can be used. For example, salt conditions  
20 from about 0.14 M to about 0.9M salt, at temperatures ranging from about 20°C to about 55°C. The presence of a hybridized complex comprising the probe and a complementary polynucleotide from the sample indicates the presence of the microbe or polynucleotide sequence in the sample.

**Methods of Eliciting an Immune Response**

Polypeptides or polynucleotides of the invention can be used to elicit an immune response in a host. Elicitation of an immune response can be used, *inter alia*, to provide model systems to optimize immune responses to microbes and to provide prophylactic or therapeutic treatment against microbial infection. Elicitation of an immune response can also be used to treat, prevent, or ameliorate a disease or infection caused by a microbe or pathogen. An immune response includes humoral immune responses and cell mediated immune responses, or a combination thereof. Preferably, an immune response is a humoral immune response.

The generation of an antibody titer by an animal against a microbe can be important in protection from infection and clearance of infection. Detection and/or quantification of antibody titers after delivery of a polypeptide or polynucleotide can be used to identify epitopes that are particularly effective at eliciting antibody titers. Epitopes responsible for a strong antibody response to a microbe can be identified by eliciting antibodies directed against microbial polypeptides of different lengths. Antibodies elicited by a particular polypeptide epitope can then be tested using an ELISA assay to determine which polypeptides contain epitopes that are most effective at generating a strong response. Polypeptides or fusion proteins which contain these epitopes or polynucleotides encoding the epitopes can then be constructed and used to elicit a strong antibody response.

A polypeptide or a polynucleotide of the invention can be administered to an animal, such as a mouse, rabbit, guinea pig, macaque, baboon, chimpanzee, human, cow, sheep, pig, horse, dog, cat, chicken, and duck, to elicit antibodies *in vivo*. Injection of a polynucleotide has the practical advantages of simplicity of construction and modification. Further, injection of a polynucleotide results in the synthesis of a polypeptide in the host. Thus, the polypeptide is

presented to the host immune system with native post-translational modifications, structure, and conformation. A polynucleotide is preferably delivered as "naked DNA."

Administration of a polynucleotide or a polypeptide can be by any means known in the art, including intramuscular, intradermal, intraperitoneal, or subcutaneous injection, intranasal, 5 mucosal, topical, and oral, including injection using a biological ballistic gun ("gene gun"). Preferably, a polynucleotide or polypeptide is accompanied by a protein carrier for oral administration. A combination of administration methods may also be used to elicit an immune response.

Administration of polypeptides or polynucleotides can elicit an immune response in the 10 animal that lasts for at least 1 week, 1 month, 3 months, 6 months, 1 year, or longer. Optionally, an immune response can be maintained in an animal by providing one or more booster injections of the polypeptide or polynucleotide at 1 month, 3 months, 6 months, 1 year, or more after the primary injection.

A composition of the invention comprising a polypeptide, polynucleotide, or a 15 combination thereof is administered in a manner compatible with the particular composition used and in an amount which is effective to elicit an immune response as detected by, for example, an ELISA, as described above. A polynucleotide is preferably injected intramuscularly to a large mammal, such as a baboon, chimpanzee, or human, at a dose of 1 ng/kg, 10 ng/kg, 100 ng/kg, 1000 ng/kg, 0.001 mg/kg, 0.1 mg/kg, or 0.5 mg/kg. A polypeptide is preferably injected 20 intramuscularly to a large mammal, such as a human, at a dose of 0.01, 0.05, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5 or 10 mg/kg. Polypeptides and/or polynucleotides can be administered either to an animal that is not infected with a microbe of interest or can be administered to a microbe-infected animal. The particular dosages polynucleotides or polypeptides in a composition will

depend on many factors including, but not limited to the species, age, and general condition of the mammal to which the composition is administered, and the mode of administration of the composition. An effective amount of the composition of the invention can be readily determined using only routine experimentation. *In vitro* and *in vivo* models described herein can be employed to identify appropriate doses. If desired, co-stimulatory molecules or adjuvants can also be provided before, after, or together with the compositions.

### **Antibodies and Their Use**

Antibodies of the invention are antibody molecules that specifically and stably bind to a microbial polypeptide of the invention or fragment thereof. An antibody of the invention can be a polyclonal antibody, a monoclonal antibody, a single chain antibody (scFv), or a part of an antibody. Parts of antibodies include Fab and F(ab)<sub>2</sub> fragments. Antibodies can be made *in vivo* in suitable laboratory animals or *in vitro* using recombinant DNA techniques. Means for preparing and characterizing antibodies are well known in the art. See, e.g., Dean, *Methods Mol. Biol.* 80:23-37 (1998); Dean, *Methods Mol. Biol.* 32:361-79 (1994); Baileg, *Methods Mol. Biol.* 32:381-88 (1994); Gullick, *Methods Mol. Biol.* 32:389-99 (1994); Drenckhahn *et al. Methods Cell. Biol.* 37:7-56 (1993); Morrison, *Ann. Rev. Immunol.* 10:239-65 (1992); Wright *et al. Crit. Rev. Immunol.* 12:125-68(1992). For example, polyclonal antibodies can be produced by administering polypeptide of the invention to an animal, such as a mouse, a rabbit, a goat, or a horse. Serum from the immunized animal is collected and the antibodies are purified from the plasma by, for example, precipitation with ammonium sulfate, followed by chromatography, preferably affinity chromatography. Techniques for producing and processing polyclonal antibodies are known in the art.



Additionally, monoclonal antibodies directed against epitopes present on a polypeptide of the invention can also be readily produced. For example, normal B cells from a mammal, such as a mouse, immunized with polypeptide of the invention can be fused with, for example, HAT-sensitive mouse myeloma cells to produce hybridomas. Hybridomas producing microbe-specific antibodies can be identified using RIA or ELISA and isolated by cloning in semi-solid agar or by limiting dilution. Clones producing microbe-specific antibodies are isolated by another round of screening. Techniques for producing and processing monoclonal antibodies are known in the art.

Antibodies, either monoclonal and polyclonal, which are directed against microbial antigens, are particularly useful for detecting the presence of microbes or microbial antigens in a sample, such as a serum sample from a microbe-infected human. An immunoassay for a microbial antigen may utilize one antibody or several antibodies. An immunoassay for a microbial antigen may use, for example, a monoclonal antibody directed towards a microbial epitope, a combination of monoclonal antibodies directed towards epitopes of one microbial polypeptide, monoclonal antibodies directed towards epitopes of different microbial polypeptides, polyclonal antibodies directed towards the same microbial antigen, polyclonal antibodies directed towards different microbial antigens, or a combination of monoclonal and polyclonal antibodies. Immunoassay protocols may be based, for example, upon competition, direct reaction, or sandwich type assays using, for example, labeled antibody. The labels may be, for example, fluorescent, chemiluminescent, or radioactive.

The polyclonal or monoclonal antibodies may further be used to isolate microbes or microbial antigens by immunoaffinity columns. The antibodies can be affixed to a solid support by, for example, adsorption or by covalent linkage so that the antibodies retain their

immunospecific activity. Optionally, spacer groups may be included so that the antigen binding site of the antibody remains accessible. The immobilized antibodies can then be used to bind microbes or microbial antigens from a sample, such as a biological sample including saliva, plaque, sputum, blood, urine, feces, cerebrospinal fluid, amniotic fluid, wound exudate, or tissue.

5 The bound microbes or microbial antigens are recovered from the column matrix by, for example, a change in pH.

Antibodies of the invention can also be used in immunolocalization studies to analyze the presence and distribution of a polypeptide of the invention during various cellular events or physiological conditions. Antibodies can also be used to identify molecules involved in passive immunization and to identify molecules involved in the biosynthesis of non-protein antigens.

10 Identification of such molecules can be useful in vaccine development.

Antibodies can be detected and/or quantified using for example, direct binding assays such as radioimmunoassay (RIA) or ELISA assays. Antibodies of the invention can be also be used to treat, prevent, or ameliorate a microbial infection, to diagnose or detect the presence or

15 absence of a microbe, and to purify an antigen or antigens that the antibody specifically binds.

#### **Compositions Comprising Polypeptides or Polynucleotides**

The invention also provides compositions comprising polypeptides or polynucleotides. Compositions of the invention preferably comprise a pharmaceutically acceptable carrier. The carrier should not itself induce the production of antibodies harmful to the host.

20 Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized, macromolecules, such as proteins, polysaccharides such as latex functionalized sepharose, agarose, cellulose, cellulose beads and the like, polylactic acids, polyglycolic acids, polymeric amino acids such as polyglutamic acid,

polylysine, and the like, amino acid copolymers, peptoids, lipitoids, and inactive, avirulent virus particles or bacterial cells.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as salts of organic acids such as acetates, propionates, malonates, or benzoates. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art. Compositions of the invention can also contain liquids or excipients, such as water, saline, glycerol, dextrose, malodextrin, ethanol, or the like, singly or in combination, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes can also be used as a carrier for a composition of the invention.

If desired, co-stimulatory molecules, which improve immunogen presentation to lymphocytes, such as B7-1 or B7-2, or cytokines such as MIP1 $\alpha$ , GM-CSF, IL-2, and IL-12, can be included in a composition of the invention. Optionally, adjuvants can also be included in a composition. Adjuvants which can be used include, but are not limited to MF59-0, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637), referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn -glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

The compositions of the invention can be formulated into ingestable tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, injectable formulations,

mouthwashes, dentifrices, and the like. The percentage of one or more polypeptides or polynucleotides of the invention in such compositions and preparations can vary from 0.1% to 60% of the weight of the unit.

The following are provided for exemplification purposes only and are not intended to  
5 limit the scope of the invention described in broad terms above. All references cited in this disclosure are incorporated herein by reference.

### **EXAMPLE 1**

*Identification Of In Vivo Induced Microbial Polypeptides From Actinobacillus*  
10 *Actinomycetemcomitans (Aa)-Infected Patients: Adsorption of LJP Serum*

To demonstrate the effectiveness of IVIAT, it was used to study *in vivo* gene induction of an oral pathogen, *Actinobacillus actinomycetemcomitans (Aa)* the putative etiologic agent of certain periodontal diseases, and, in particular, localized juvenile periodontitis (LJP) (Slots & Ting, *Periodontol. 2000*, 20:82-121 (1999)). *Aa* is well suited for this purpose since it typically  
15 evokes a strong humoral immune response in periodontitis patients (Kinane *et al. Periodontol. 2000*, 20:289 (2000)) and there is currently no reliable animal model with which to study this disease.

Serum samples were obtained from 20 periodontitis patients identified by family history and clinical and radiographic examinations. Microbiological and immunological analyses  
20 indicated a role for *Aa* as the etiologic agent in each of these cases. See, e.g., Ebersole *et al. J. Clin. Immunol.* 3:321 (1983); Tsai *et al., J. Clin. Periodontol.* 8:338 (1981). These sera were tested individually by a modified ELISA procedure (Ebersole, *J. Periodont. Res.* 15:621 (1980)) using French-press disrupted cell extracts of *Aa* strain HK1651 (ATCC Accession No. 700685)

to verify that they retained their reactivity to *Aa*. HK1651 is a member of the clonotype distinguished by a 530 bp deletion in the promoter region of the leukotoxin gene, and which has been characterized as being particularly virulent and contagious. Haubek *et al. J. Clin. Microbiol.* 34:1576 (1996). Equal amounts (0.05 ml) of each patient's serum were then pooled.

- 5 Pooled sera from patients were utilized in this instance to maximize the spectrum of antibodies reactive with *Aa* epitopes, including immunodominant ones.

Antibodies in the pooled serum that were reactive with proteins made by *Aa* HK1651 during *in vitro* cultivation were eliminated by repeated adsorptions using the methods of Brady *et al., J. Infect. Dis.* 158:965-972 (1988). Adsorptions were performed using both whole cells and  
10 cell extracts immobilized on nitrocellulose. Briefly, 500  $\mu$ l of the pooled sera were subjected to five successive adsorptions against *in vitro* grown whole cells of *Aa* strain HK1651. The cells were grown in BHI broth at 37°C in ambient atmosphere enriched with 5% CO<sub>2</sub>. Each adsorption consisted of an overnight incubation of the pooled sera with approximately 10<sup>11</sup> bacteria in 100  $\mu$ l phosphate buffered saline (PBS, pH 7.2) containing 0.02% sodium azide with  
15 mild agitation at 4°C. The serum was further adsorbed by incubation overnight at 4°C with a nitrocellulose membrane (10 cm diameter) saturated with HK1651 extracts prepared by French-press treatment of 10<sup>11</sup> *in vitro* grown *Aa* cells. A final adsorption step was carried out using the same extract which was heat denatured in a boiling water bath (10 min.) before immobilization on nitrocellulose in order to expose additional immunoreactive epitopes.

- 20 To evaluate the efficiency of the adsorption steps, French-press cell extracts of HK1651 were immobilized in microtiter wells and, following a modified ELISA procedure (Ebersole, 1980), were reacted with serial dilutions of serum samples taken at different points in the adsorption process. Peroxidase-conjugated goat anti-human affinity-purified immunoglobulin

(ICN Cappel; Aurora OH), reactive with all classes of human immunoglobulins, was used to determine the reactive antibody titers. As shown in Figure 2, the successive adsorptions essentially removed all of the antibodies reactive with *in vitro* grown HK1651. In preliminary studies it was determined that it was unnecessary to adsorb the serum with the *Escherichia coli* host strain since it produced a relatively low background signal.

## **EXAMPLE 2**

### *Expression Library Construction And Screening With LJP Serum*

Genomic expression libraries of HK1651 in PET30abc were prepared in *E. coli* strain BL21(DE3) (Novagen, Madison, WI). Briefly, genomic DNA purified from *Aa* strain HK1651 was partially digested with *Sau3A* to optimize the production of 0.5 to 1.5 kb fragments which were purified by agarose gel electrophoresis. The fragments were ligated into the multiple cloning site of pET30a inducible expression system (Novagen; Madison, WI) that had been digested with *Bam*HI, dephosphorylated with calf intestinal phosphatase (New England Biolabs, Beverly, MA), and then transformed into *Escherichia coli* strain BL21(DE3) (Novagen). Similar libraries were also constructed with pET b and c.

The resulting HK1651 genomic expression library was serially diluted and plated on brain-heart infusion (BHI) medium containing kanamycin (50 µg/ml) to generate plates containing approximately 500 colonies per plate. These colonies were replicated using sterile velvet onto duplicate BHI plates containing kanamycin and IPTG (1 mM) and incubated for 5 hours at 37°C to induce expression of the cloned polynucleotides. To lyse the bacteria the colonies were exposed to chloroform vapors for 15 min. and overlaid with nitrocellulose membranes for 15 min. at room temperature. The membranes were carefully removed and blocked with 5% non-fat skim-milk solution in PBS at pH 7.2 containing 0.5% Tween-20 (PBS-

Tween™). The colonies were first probed with a 1:10,000 dilution of adsorbed serum (based on the results shown in Figure 2) overnight at 4°C and then with a 1:10,000 dilution of goat anti-human peroxidase conjugated antiserum under the same conditions. Reactive colonies were detected using the ECL kit and Hyperfilm™ ECL (Amersham Pharmacia Biotech, Piscataway N.J.). X ray film exposure times were adjusted to optimize signal to noise.

Figure 3 demonstrates the reactivity observed when replica colony blots were probed in parallel with either unadsorbed or adsorbed pooled serum. Plates containing appropriate numbers of colonies were replicated onto medium with IPTG to induce expression of the cloned fragments. The colonies were lifted onto duplicate membranes, which were then reacted with either the pooled unadsorbed (panel A) or pooled adsorbed (panel B) sera. Reactive clones were visualized by probing with peroxidase-conjugated goat anti-human immunoglobulin as a secondary reagent followed by development with a chemiluminescent substrate and autoradiography.

The majority of clones reacted with the pooled unadsorbed serum, indicating that periodontitis patients had produced antibodies that recognized a broad array of *A. actinomycetemcomitans* proteins. Only a small subset of colonies tested (13 out of 20,000 screened) reacted with the pooled adsorbed serum. These IVIAT clones were isolated.

Potential bias created by using a single restriction enzyme is eliminated by using a second restriction enzyme (*e.g.*, *MboI*), DNA sheared by a HydroShear™ apparatus (GeneMachines, San Carlos CA), or sonically sheared DNA to create a second, independent expression library in, for example, pET30abc. In the latter case, purified HK1651 DNA at a concentration of approximately 1 µg/µl is treated on ice with 3 second resonance frequency bursts of sonication using a Microson Ultrasonic Cell Distruptor (Heat Systems-Ultrasonics, Inc.). Samples taken at

each time point are analyzed by agarose gel electrophoresis to determine the conditions required to optimize production of 0.75 to 1.5 Kb fragments. These conditions are employed in a large scale experiment to generate sufficient starting material for library construction. Terminal overhangs are removed with Klenow and dNTPs and the resulting blunt end products are ligated  
5 into the dephosphorylated *EcoRV* restriction site in the pET30abc multiple cloning site. The vector library is then be transformed into *E. coli* host strain, BL21 (DE3), with clones selected on BHI/kanamycin medium as previously described. Plates containing appropriate numbers of colonies are probed with the adsorbed serum and reactive colonies are detected using peroxidase-labeled goat anti-human immunoglobulin. Reactive colonies are isolated and their vector DNA  
10 purified.

### **EXAMPLE 3**

#### *Characterization Of Polynucleotide Clones Reactive With Adsorbed LJP Serum*

Sequencing of the cloned inserts in both directions indicated that the 13 recovered clones consisted of 8 unique clones. See SEQ ID NOs:1-8. MacVector v.6.0.1 was used to identify  
15 open reading frames using both Fickett's and start/stop codon methods. In every instance, potential open reading frames were present on the cloned inserts oriented in both the same and opposite direction relative to the pET promoter (Table 1).



Table 1. Characterization of the IVIAT open reading frames. (\* Putative ribosome binding site.)

IVIAT clone	Schematic representation of ORFs	IPTG inducibility
47		+
26		+
30		+
37		+
39-1		+
46-2		+
47-4		-
58-3		-

In order to determine which of these was most likely to be the one encoding the antigen reactive with the adsorbed serum, a colony lift method was first used to determine if the level of expression of the antigen was regulated by IPTG. Briefly, Each IVIAT clone was grown  
5 overnight at 37°C in BHI broth containing kanamycin, the cells were concentrated 200-fold, and 1 µl of each culture was spotted on BHI agar plates containing kanamycin with or without IPTG. Following incubation of the plates at 37°C for five hours, the colonies were partially lysed with chloroform vapors, lifted onto nitrocellulose membranes, and reacted with the adsorbed serum. Two negative controls were included in this IPTG induction assay, namely pET30a/BL21(DE3)  
10 with no cloned insert and a randomly selected clone (IVIAT-47) which contained a cloned DNA insert, but was non-reactive with the adsorbed serum.

This assay demonstrated that 6 of the 8 IVIAT clones expressed immunoreactive proteins that were induced by IPTG. *See* Table 1 and SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19). Therefore, their open reading frames of  
15 interest were likely cloned in the same orientation as the endogenous pET30 promoter. Their translation may either be initiated at the pET30 ribosome binding site or at their own ribosome binding site. We further examined 6 of the 11 possible open reading frames found in the IPTG inducible clones (Table 2). Each of these open reading frames was individually amplified by PCR and subcloned so that it was in-frame and under control of the pET30EkLIC (Novagen,  
20 Madison, Wisconsin) IPTG regulated promoter. *See* SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. Reactivity with the adsorbed serum and IPTG inducibility of these subclones was confirmed using the colony lift method used previously (Table 2). Four polynucleotides with demonstrable homology were found, including

one (Aa2004) that is related to a known virulence factor (*hlpA*) in the closely related organism, *Haemophilus influenza*. There are three examples of polynucleotides with homology to genes encoding proteins with known function but with previously undemonstrated relevance to pathogenesis (Aa2005; Aa2009; Aa20011). Two polynucleotides were found with no homologues identified to date (Aa2008; Aa20010). These types of *in vivo* induced polynucleotides may well represent entirely novel virulence factors of *Aa*. Interestingly, none of the open reading frames thus far identified on the clones isolated by this invention show homology to genes with known housekeeping functions. The reason for this difference is uncertain, but this represents an important additional advantage of the method described herein.

10

#### **EXAMPLE 4**

##### *Verification of Specific In Vivo Expression*

It is possible to directly verify that *in vivo* induced antigens identified by IVIAT are actually expressed by the pathogen when it is growing at the site of infection in the human host. To accomplish this, a method to directly probe biological specimens taken from disease sites of infected patients was developed. The first step in this process involved optimization of recombinant protein production by the subclones obtained in the previous step. Clone Aa2008 contains a 495 bp open reading frame (SEQ ID NO:13) for a hypothetical protein subcloned into pET30EkLIC (Table 2). Including the 5' and 3' sequences added by the vector, the molecular weight of the translated product should be approximately 28 Kda. Aa2008 was incubated in BHI broth containing kanamycin and IPTG (5 mM). Samples of the culture were removed at hourly intervals and analyzed by SDS-PAGE to determine the incubation time required for maximum production of the cloned protein. A major band (not seen in pET30EkLIC/BL21(DE3) control samples) was found that had the expected molecular weight. Based on the results obtained,

inclusion body preparations (Harlow & Lowes Eds., *Antibodies, a Laboratory Manual*, (CSHL

Press, Plainview, NY, 1988)) were made from 10 ml batch cultures of Aa2008 incubated for 3 hours with IPTG. SDS-PAGE and Western blot analysis was performed to confirm that the overexpressed protein was reactive with the adsorbed serum used in the original screening. The  
5 reactive band was excised from the Coomassie stained gel and used to raise polyclonal antibodies in mice.

Three female Balb/C mice are used per antigen. The 6-8 week old mice were injected subcutaneously (sc) with the purified polypeptide, using a concentration of 20, 50, or 100 µg/mouse. RIBI MPL + TDM emulsion was used an adjuvant. Two weeks later, the  
10 immunization protocol was repeated. Approximately 10 days after the second immunization, a small amount of blood was taken from the tail vein of each mouse, and assayed by ELISA to evaluate the response to the immunogen. Where the mice were responding well (*i.e.*, detection at serum dilution greater than 1:500), one more sc injection was given 3-4 weeks after the second immunization, and four days before the scheduled hybridoma fusion. A mouse myeloma cell line  
15 (Sp2/0) was cultured for several days prior to the scheduled fusion. Log phase cells were counted and washed.

The mouse exhibiting the best serum titer was anesthetized and bled out. The spleen was removed. The blood was clotted and spun down and the serum saved to be used as a positive control in the subsequent assays. The spleen cells were flushed out of the spleen capsule and  
20 separated into single cells, washed, and counted. Spleen cells were combined with Sp2/0 cells at a 7:1 ratio. Cells were pelleted by centrifugation. PEG 1500 at a concentration of 50% was slowly added to the cell pellet and then diluted. Cells were spun down and the pellet resuspended in HAT selective medium containing 20% horse serum and 25% conditioned media.

Cells were plated into 96 well plates at a concentration of  $2.5 \times 10^5$  cells/well. Cells were grown in a 37°C incubator with 7% atmospheric CO<sub>2</sub>. After several days, colonies of hybridoma cells were visible. Feeding (removing and replacing approximately 50% of the total volume of growth medium) was performed at day seven and again at day nine or ten. When the hybridoma cells were nearly confluent in each well, a portion of the supernatant was removed and assayed by ELSIA to detect the presence of the desired antibody. The cells in the wells that tested positive on the primary screen were transferred to a 24 well plate, and the supernatant tested again by ELISA. The antibody activity and preliminary heavy chain isotype of each mass culture was determined and the wells that scored positive during this secondary round of screening were transferred to 6 well plates. At this point the cells were designated as mass cultures. Each mass culture chosen was cultured briefly in 6 well plate wells in order to freeze two vials of cells. Supernatant was saved for future expanded screening. The best mass cultures were selected for ascites production. Ascites were induced by intraperitoneal injection into one female Balb/C mouse, which was monitored daily for 7 days. The ascitic fluids were collected every day for 3 days (for a final volume of ca. 10ml).

Ascites fluid were filtered and circulated over a protein A affinity column to affinity purify IgG. The polyclonal antibodies were washed and eluted with 0.1 M glycine, pH 3.0, followed by neutralization with Tris, pH 9-10. The recovered antibodies were concentrated, and the buffer exchanged to 1 x PBS with 0.02% azide. The yield and quality of the antibodies were evaluated spectrophotometrically (280 nm) and by SDS-PAGE, respectively.

The purified IgG was shown to react in western blots with a protein of the appropriate size in extracts and inclusion body preparations made from clone Aa2008. The antibodies were

labeled directly with fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR, USA).

A murine monoclonal antibody specific for *Aa*, Mab 13(10B2), was labeled with Texas-red (TR).

Subgingival plaque from patients with LJP was sampled at the time of their diagnosis.

Sterile paper points were inserted into mesial, buccal and distal sites of all four first molars that

5 had significant (greater than or equal to 5 mm) bone loss. The points were immediately immersed in ice-cold BHI broth containing 50% glycerol, and after removing a small sample for microbiological identification of *Aa*, they were quick frozen in a dry ice/ethanol bath and stored at -80°C. Samples which were confirmed to contain *Aa* by growth on selective medium were used to directly demonstrate the presence of Aa2008.

10 Plaque samples and matched *in vitro* grown cells were assayed by immunofluorescence microscopy (Fig. 4). Briefly, plaque samples were thawed on ice, homogenized by brief vortexing at 4°C, and immediately heat-fixed on microscope slides. Plaque samples and matched *in vitro* grown *Aa* isolates taken from the selective medium were probed in parallel with FITC-labeled monospecific antibody directed against antigen Aa2008 and the TR-labeled *Aa*-specific  
15 monoclonal antibody. Samples were examined by fluorescence microscopy (excitation wavelength 488 nm). Double color analysis, using matched differential emission filters, was performed to identify individual cells that were simultaneously labeled with FITC and TR. Bacterial cells in plaque that reacted with TR-labeled anti-*Aa* monoclonal antibody (Figure 4A) also reacted with FITC-labeled anti-Aa2008 antibody (Figure 4B) and these signals were found  
20 to colocalize (Figure 4C). In contrast, matched *in vitro* grown *Aa* reacted with the TR-labeled anti-*Aa* monoclonal antibody (Figure 4D), but not with FITC-labeled anti-Aa2008 antibody (Figure 4E) and therefore no colocalization was observed (Figure 4F). These results provide direct evidence that *Aa* expresses antigen Aa2008 exclusively during *in vivo* growth.

Each anti-IVIAT antibody may independently screened against all of the plaque samples collected (3 samples/tooth x maximum 4 teeth/patient x 10 patients  $\leq$  120 samples) in order to optimize the identification of *Aa* growing under environmental conditions appropriate for expression of the *in vivo* induced antigen. *Aa*-deficient samples are included to serve as an additional negative control for the species specific antibody and to identify possible cross-reacting antigens presented by other species that may be present in plaque. Based on our previous studies, we expect that approximately 75% of samples will contain measurable numbers of *Aa*. Of the three most likely outcomes, there will be instances where cells react with Texas red-labeled anti-*Aa* antibody and one or all of these cells also react with a FITC-labeled anti-IVIAT antibody. This indicates that particular antigen is produced by *Aa* during growth in a site of infection. There is no requirement for all of the *Aa* cells in a sample to react with the anti-IVIAT antibody since there are likely to be numerous microenvironments present at each sampling site. As a second possible outcome, there may be instances where none of the cells which react with the Texas red-labeled anti-*Aa* antibody also react with a particular FITC-labeled anti-IVIAT antibody. This indicates that none of the plaque samples were taken from sites presenting the necessary environmental stimulus. Clearly, by using a large number of plaque samples taken from patients in different stages of infection, we hope to reduce the occurrence of this possibility. This result may also indicate that the *in vivo* induced antigen was expressed but not surface localized. Thus, in situations where a particular anti-IVIAT antibody does not react with *Aa* cells in any of the plaque samples, one may examine intra- and extra-cellular compartments for the antigen. This is done simply by examining plaque sample homogenates using a capture ELISA method: microtiter wells are coated with unlabeled anti-IVIAT antibody, reacted with homogenate, and then probed with FITC-labeled anti-IVIAT antibody according to

Current Protocols in Immunology, John Wiley & Sons, NY (1998). The presence of reactivity,

as measured by fluorescent light emission, is then correlated to the presence of *Aa* as determined by prior microbiological, ribotyping and immunological analyses of the plaque samples. As another possible outcome of interest, plaque samples may contain cells that react with a particular FITC-labeled anti-IVIAT antibody but which do not react with Texas red-labeled anti-*Aa* antibody. These are likely to represent instances where plaque samples contain other bacterial species which produce a cross-reacting antigen. However, it is also possible that these cells belong to a strain or variant of *Aa* which does not react with the species anti-*Aa* antibody. In these instances, previous culturing data along with ribotyping verification will help us to distinguish between these possibilities.

As an alternative approach to the microscopy methods described above, one may use FITC-labeled anti-IVIAT antibodies and a fluorescence-activated cell sorting system (e.g., FACSsort, Becton Dickinson; Franklin Lanes, NJ). Recent reports have demonstrated the technical feasibility of using automated cell sorters for separating bacteria (Valdivia & Falkow, *Science*, 277:2007 (1997); Handfield & Levesque, *FEMS Microbiol. Rev.* 23:69-91 (1999). In addition, FACS can be used to effectively separate *Aa* from a mixed bacterial population using the green fluorescence protein (GFP) as a gene marker. In this study, a strain of *Aa* (SUNY 465) expressing the GFP under the leukotoxin *ltx* promoter was separated from *Aa* strain Y4 with the FACSsort.

## **EXAMPLE 5**

### *Screening Patients for Reactive Antibodies*

Once IVIAT has identified and confirmed an *in vivo* induced antigen, the purified antigen can be used to screen the sera of individual patients for reactive antibodies. Western blots of the



inclusion body preparation of Aa2008 were probed using individual, unadsorbed sera from 20 periodontitis patients and 9 control subjects (Figure 5). Densitometric analysis of Western blots showed that the patient group demonstrated significantly ( $p < 0.05$ , independent student's T-test) higher reactivity with the recombinant Aa2008 protein ( $3.75 \pm 1.12$ , mean  $\pm$  standard deviation) than a control group that did not have periodontitis ( $2.79 \pm 0.98$ ). These data suggest that Aa2008 is immunogenic in periodontitis patients, and confirms the conclusion drawn from fluorescence microscopy of plaque samples that this represents an *in vivo* expressed polynucleotide.